Selective Recovery of *Xanthomonas* spp. from Rice Seed

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ABSTRACT

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A semiselective medium, called XOS, was developed to isolate *Xanthomonas oryzae* pv. *oryzae* and *X. o. oryzicola* from rice seed. The medium contains sucrose, peptone, monosodium glutamate, calcium nitrate, potassium phosphate, iron EDTA, cycloheximide, cephalexin, kasugamycin, methyl violet 2B, and agar. Recovery of *X. o. oryzae*, *X. o. oryzicola* and nonpathogenic *Xanthomonas*-like bacteria on XOS basal medium ranged from 98 to 162% (mean 121%) of the recovery on nutrient glucose agar after incubation at 28 C for 4 days. Addition of the selective inhibitors decreased recovery of non-*Xanthomonas* saprophytes in rice seed extracts by over 53%, compared to XOS basal medium. Several saprophytic bacteria common to some Chinese seed lots were not significantly reduced; however, the growth of such bacteria was decreased enough to allow detection of the slower growing xanthomonads. Recovery of different pathogenic strains of the rice xanthomonads varied significantly on XOS. Detection of *Xanthomonas* spp. from seed was dependent on partial maceration of seed and incubation of the resulting extract at 5 C for 2 h. Extracts from rice seed collected from asymptomatic plants in California and China and from plants with symptoms of bacterial leaf blight in China were plated on XOS, and colonies suggestive of *Xanthomonas* were isolated. Various identification assays indicate that these strains belong to the genus *Xanthomonas*; however, the strains were nonpathogenic when inoculated on a susceptible rice cultivar. XOS is quantitatively and qualitatively superior to other available media for isolation of *Xanthomonas* spp. from rice seed.

Additional keywords: rice bacterial blight, seed assay.

Bacterial leaf blight and leaf streak, incited by *Xanthomonas oryzae* pv. *oryzae* and *X. o. oryzicola* (25), respectively, are significant worldwide constraints to the production of rice (*Oryza sativa* L.). Before 1970, leaf blight was confined to Asia (18), and in China it was found only in a few localized rice-growing areas (H. Ye, *unpublished data*). Since then, the disease has been reported throughout most rice-growing areas of Asia (6,17,18). The disease has now been reported in Central and South America (15), Australia (1), and Africa (20). Jones et al (14) recently reported strains of *X. o. oryzae* with decreased virulence in Texas and Louisana. These atypical strains have not been found in other major rice-growing regions in the United States. Although environmental conditions needed for disease development most likely occur, typical *X. o. oryzae* strains have not been found in the United States. Leaf streak is not as widely distributed as leaf blight but causes significant losses in China and southeast Asia (18). It also has not been reported from the United States or other temperate regions (18).

The leaf streak and leaf blight pathogens are similar biochemically but *X. o. oryzae* is slower growing and more fastidious than *X. o. oryzicola*. The symptoms they induce on susceptible rice are easily differentiated, except at an advanced stage (18). There is still significant uncertainty regarding the source of inoculum and the most effective methods of control for both diseases. Although both pathogens are assumed to be seedborne (16,18), seed transmission has not yet been proven for either pathogen. Recently, yellow-pigmented nonpathogenic *Xanthomonas*-like bacteria have been isolated from rice in Texas (2,14). The occurrence of nonpathogenic *Xanthomonas*-like bacteria from rice plants and seed may be widespread and contribute to the thought that *X. o. oryzae* is seedborne. This may have a significant impact on the application of regulatory procedures already implemented to limit the introduction of pathogenic strains (4). Regulations should differentiate nonpathogenic and pathogenic xanthomonads in rice seed. A reliable detection method is needed to define the ecology of xanthomonads in rice seed as well as to determine the extent and significance of nonpathogenic *Xanthomonas*-like strains in rice seed.

Detection is dependent on the integration of procedures to effectively release target bacteria from seeds and other plant tissues and the availability of a medium that efficiently segegrates target strains from contaminating microorganisms. For *X. o. oryzae* and *X. o. oryzicola* this has proven difficult, in part, due to their slow growth rate (especially *X. o. oryzae*) and their poor competitive ability relative to associated saprophytic bacteria. Herein we describe a protocol to efficiently release *Xanthomonas* spp. from rice seed and to culture them on a semiselective agar medium.

MATERIALS AND METHODS

Source of strains and naturally infested rice seed. Bacterial strains used in these studies are listed in Table 1. All *Xanthomonas* strains were isolated from rice seed or leaf tissue and tested for pathogenicity by inoculation of leaves of 25-day-old rice seedlings cv Calmochi as described below. Predominant saprophytic bacteria from several Asian and American rice seed lots also were utilized in developing the semiselective medium.

Development of semiselective medium. Dilution plating of pure cultures of various pathogenic and nonpathogenic *Xanthomonas* strains was used to evaluate individual components at different concentrations and to test complex mixtures of ingredients for the basal medium (27). Strains were grown in nutrient glucose broth (23) in a New Brunswick environmental incubator-shaker at 100 rpm for 24 h at 28 C. Cell concentrations were standardized with a colorimeter and diluted 10^{-5} and 10^{-6} with sterile saline (23). Aliquots (0.1 ml) were plated onto nutrient glucose agar (NGA) (23) and the test media. Plating efficiency (recovery) was calculated as number of colony-forming units (cfu) on the test medium per number of colony-forming units on NGA × 100, after incubation at 28 C for 4 days. Nutritional sources included sucrose, glucose, trehalose, rice starch, maltose, peptone, tryptone,

nutrient broth, yeast extract, and casein hydrolysate, sodium glutamate, methionine, cystine, cysteine, proline, KH_2PO_4, K_2HPO_4, $Ca(NO_3)_2$, NH_4Cl, $NH_4H_2PO_4$, $MnSO_4$, $MgCl_2$ $6H_2O$, $CaCl_2$, KBr, NaCl, $CaCO_3$, $MgSO_4 \cdot 7H_2O$, $FeSO_4$, and Fe(EDTA). The resulting basal medium was termed XOS basal medium.

Thirty-three inhibitors and 18 dyes were tested for their differential ability to suppress saprophytic bacteria of rice seed without significantly diminishing the recovery of the target strains. These included the inhibitory chemicals ampicillin, carbenicillin, cephalexin, cephalothin, chloramphenicol, Ca-pantothenate, bacitracin, erythromycin, gentamicin sulfate, kanamycin, kasugamycin, lithium chloride, lithium nitrate, lithium sulfate, Na-dichromate, nalidixic acid, novomycin, neomycin sulfate, sodium azide, novobiocin, pimaricin, penicillin G, potassium tellurite, polymycin B, potassium cyanide, pyridoxine HCl, rifampicin, nicotinic acid, nitrofurantoin, streptomycin sulfate, tobramycin, thimerosal, trimethoprim, and the dyes, alcian blue, brillant blue R, bromcresol purple, bromcresol green, bromothymol blue, bromophenol blue, congo red, crystal violet, Evans blue, matrius yellow, methyl green, methyl violet 2B, night blue, neutral red, phenol red, trypan blue, safranin, and xylene cyanol. Cycloheximide, Benlate (DuPont Chemical Co., Wilmington, DE) and chlorothalonil (Diamond Shamrock Chemicals Co., Chardon, OH) were utilized to inhibit fungi. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except where noted.

Difco paper concentration disks (0.1-ml capacity) were soaked in a known amount of the inhibitor, placed on XOS basal medium as described, and after 1 h the plates were oversprayed with a suspension of the test strain (10^8 cfu/ml) (19,26). Preliminary screening was based on the diameter of the zone of inhibition of bacterial growth after 3–4 days at 28 C.

Secondary screening was done by incorporating selected inhibitors and dyes alone and then in combination into XOS basal medium. Recovery of saprophytic bacteria and target bacteria from pure and mixed cultures was determined after a 4- to 5-day incubation period at 28 C. All assays were repeated at least three times, using 6–10 plates per experiment. The resulting medium was named XOS. Recovery of xathomonads on XOS was compared by dilution plating assays with the following media: NGA (23), SUWA (24), Wakimoto's WFP medium (18), nutrient broth yeast extract agar (NBY) (26), yeast-dextrose-calcium carbonate agar (YDC) (26), peptone sucrose agar (PSA) (12), and MXO (11). Finally, dilution plating of pure cultures of representative nonpathogenic *Xanthomonas*-like strains from seed, Texas strains of nonpathogenic *Xanthomonas* spp. from leaves, low virulence *X. o. oryzae* strains from Texas, and virulent *X. o. oryzae* and *X. o. oryzicola* strains was done to compare the plating efficiencies of XOS and MXO.

Extraction of bacteria from seeds. Several methods (21) including passive soaking and grinding were initially evaluated for release of the target bacteria from seeds. None were efficient and reliable. The use of a specialized laboratory blender (Stomacher, model STO-400, Tekmar Co., Cincinnati, OH), as used for *Clavibacter michiganensis* subsp. *michiganensis* (7), was then evaluated in conjunction with seed soaking and found to be effective for target extraction. This apparatus was subsequently used in assays to determine the optimum digestion and soaking times and temperature. The final conditions were defined based on optimium recovery of the target bacteria with minimium interference from contaminating bacteria. The amount of seed used in the extraction procedure is only limited by the capacity of the Stomacher. For our tests, due to the restricted availability of known contaminated seed, 25 g of seed (approximately 1,000 seeds) were typically used. Seeds were washed thoroughly in sterile tap water for 10 min, to minimize interference from debris, then placed in a 18- × 30-cm heavy duty polyethylene bag (Tekmar Co.) with 50 ml of sterile phosphate-buffered saline solution (PBS) (0.1 M sodium phosphate, 0.85% sodium chloride, pH 7.2). The bag was placed in the Stomacher for digestion times of 15, 20, or 30 s. After maceration in the Stomacher, contents of the bag were transferred to a 500-ml flask and incubated an additional 2–24 h at 5 or 25 C. Two samples of seeds were used in the extraction assays.

Recovery of *Xanthomonas* strains from contaminated seeds. Seeds were collected from asymptomatic rice plants in California and China and from rice plants with severe symptoms of leaf blight in China. However, tests were not done initially on xantho-

TABLE 1. Source and percentage of recovery of *Xanthomonas oryzae* pv. *oryzae*, *X. o. oryzicola*, and *Xanthomonas*-like strains on XOS basal medium

Strain	Source	Recovery[z] (%)
Xanthomonas oryzae pv. *oryzae*		
TJ-13	Z. Zhang, Hebei province, China	98
BJ-4	Z. Zhang, Hebei province, China	110
HLT-69	Z. Zhang, Heilonjing province, China	104
PXO 61	T. Mew, IRRI, Philippines (strain B748, N. W. Schaad)	103
PXO 99	T. Mew, IRRI, Philippines (strain B752, N. W.Schaad)	104
PXO 89	T. Mew, IRRI, Philippines (strain B794, N. W. Schaad)	114
LN 844	Z. Zhang, Liaoning province, China	114
PXO 721	T. Mew, IRRI, Philippines (strain B791, N. W. Schaad)	112
X1-8	C. Gonzalez, Texas	111
HN-31	Z. Zhang, Henan province, China	123
PXO 79	T. Mew, IRRI, Philippines (strain B792, N. W. Schaad)	116
HX-42	Z. Zhang, Ningxia province, China	122
KS 6-6	Z. Zhang, Jiangsu province, China	120
ZHE-173	Z. Zhang, Jiangsu province, China	118
SX-73	Z. Zhang, Shaanxi province, China	125
JS 262	Z. Zhang, Hebei province, China	148
PXO 86	T. Mew, IRRI, Philippines (strain B793, N. W. Schaad)	149
PXO 112	T. Mew, IRRI, Philippines (strain B795, N. W. Schaad)	153
HB-17	Z. Zhang, Hebei province, China	162
X. oryzae pv. *oryzicola*		
S44	H. Ye, Sichuan, China	112
S1358	H. Ye, Sichuan, China	122
Xanthomonas-like		
X1-1	C. Gonzalez, Texas	114
X1-9	C. Gonzalez, Texas	120
NP1	D. Roth, Chinese rice seed	112
NP2	D. Roth, California rice seed	135

[z]Recovery (plating efficiency = number on XOS basal medium of colony-forming units per number of colony-forming units on nutrient glucose agar × 100. Figures were calculated from the mean colony numbers per plate from 6–10 plates per strain.

monad-like strains isolated from seed to confirm pathogenicity. Recovery of target colonies on XOS and MXO was determined from extracts of seed collected from infected plants and from seed extracts to which *X. o. oryzae* or *X. o. oryzicola* cells were added (artificially infested seed extracts). Cells were added to the extract from 1,000 seeds of a pathogen-free lot, such that plating of 0.1-ml aliquots of a 10^{-2} dilution would yield approximately 10 cfu/plate, simulating a low inoculum density in seed.

In addition, rice seed, collected from plants in China with characteristic symptoms of severe leaf blight, was diluted with xanthomonad-free seed to give mixes containing 1, 10, or 50% contaminated seed. Extracts were prepared and assayed as described above to determine the recovery of xanthomonads and non-*Xanthomonas* bacteria on the XOS basal medium, XOS, and MXO.

Identification and pathogenicity testing of target bacteria. Colonies suspected of being *Xanthomonas* were initially identified on XOS based on typical morphology and color compared to known *X. o. oryzae*. Gram staining was done as described (23). Growth of strains was compared by streaking onto YDC, NBY, and PSA media. Bacterial pigments were methanol-extracted from log phase cells grown in YDC broth. Xanthomonadin pigments were detected and identified with scanning spectrophotometry and thin-layer chromatography (13). High molecular weight RNA was isolated from the xanthomonad strains HN-31, LN-844, PXO61, X1-8, NP1, NP2, X1-1, S44, and S1358 and from *Erwinia herbicola* strains RS1, RS2 (both from rice seed), *E. chrysanthemi*, *Pseudomonas syringae* pv. *phaseolicola*, *Pseudomonas* spp. from rice seed, and *P. s. tomato* according to DeParasis and Roth (5). RNA (2 μg) was blotted onto Hybond N membranes (Amersham, Arlington Heights, IL) using a Minifold II slot blot system (Schleicher & Schuell, Keene, NH) and crosslinked by exposure to ultraviolet light for 30 s at a distance of 4 cm. Nucleic acid hybridizations were done according to Bragg and Bollon (3) using a *Xanthomonas*-specific DNA probe based on a subset of 16s rRNA sequences (5; H. Ye and D. A. Roth, *unpublished data*). Routine pathogenicity testing was done by inoculating 25-day-old rice seedlings cv Calmochi by puncturing leaves with a needle dipped into inoculum from a 24-h YDC agar culture. *X. o. oryzae* strain LN-44 was used as a positive control. Plants were incubated in a growth chamber at 28 C. At least three plants were tested per strain and symptoms were evaluated 7 and 14 days post-inoculation. Virulence was determined based on lesion length, and the pathogen was re-isolated from the lesion margin.

RESULTS

Development of a semi-selective medium. Recovery and colony diameter of 19 strains of *X. o. oryzae* and two strains of *X. o. oryzicola* were used as the basis to select ingredients and concentrations for the basal medium. Optimal recovery and colony size of these strains were obtained with sucrose as the carbon source. Additional essential components for growth were identified based on extensive comparative testing using representative strains. The resulting medium, called XOS basal medium, consisted of 20 g of sucrose, 2 g of peptone, 5 g of monosodium glutamate, 0.2 g of $Ca(NO_3)_2$, 2 g of K_2HPO_4, 1 mg of Fe(EDTA), and 17 g of agar per liter. The pH of the medium was 6.8–7.0. Variation in recovery and colony diameter was observed among *X. o. oryzae* strains on XOS basal medium. Less variation was observed between the *X. o. oryzicola* strains. Recovery of *X. o. oryzae*, *X. o. oryzicola* and xanthomonad-like strains on XOS basal medium ranged from 98 to 162, 112 to 122, and 112 to 135% of recovery on NGA, respectively (Table 1). Mean recovery of *X. o. oryzae*, *X. o. oryzicola*, and nonpathogenic *Xanthomonas*-like strains on XOS basal medium compared to NGA was 121%.

Of the various inhibitors and concentrations tested, cycloheximide (100 mg/l), cephalexin (20 mg/l), kasugamycin (20 mg/l), and methyl violet 2B (0.3 μg/ml) significantly suppressed most representative seedborne microorganisms with minimium reduction in growth of target strains. The final medium including inhibitors and antibiotics is termed XOS. Xanthomonad colonies on XOS were visible (1 mm in diameter) after 24–30 h and were light yellow, mucoid, round, and smooth. Mean recovery on XOS medium of three *X. o. oryzae* and two *X. o. oryzicola* strains was reduced by approximately 13% in comparison to XOS basal medium after 4 days at 28 C (Table 2). *X. o. oryzae* and *X. o. oryzicola* grew poorly on MXO medium. Mean recovery of *X. o. oryzae* and *X. o. oryzicola* strains on MXO medium was reduced 79% of that on XOS basal medium (Table 2). In three other assays, recovery of nontarget bacteria associated with rice seed from pure and mixed cultures was decreased 44, 48, and 68% (mean 53%) on XOS compared to XOS basal medium. The two predominant saprophytic bacteria from rice seed extracts growing on XOS were *E. herbicola* and a *Pseudomonas* spp. Although recovery of these two saprophytes was not dramatically reduced on XOS, growth was sufficiently suppressed to allow even the slowest growing target strains to be observed.

Extraction of target bacteria from infested rice seed. The most efficient time of extraction of seed in the Stomacher blender for release of target bacteria was 20 s. At this time, approximately 80% of the seeds were broken; at 30 s or more, additional seed constituents that interfered with recovery of the target organisms on XOS were released. At 15 s only 50% of the seeds were broken and, thus, recovery of xanthomonads was significantly impaired.

After extraction, incubation of the extracts at 25 C substantially increased interference from saprophytic bacteria, particularly *E. herbicola*, over incubation at 5 C. No significant differences in recovery of target bacteria were observed between 2 and 24 h of soaking, whereas a number of non-xanthomonad bacteria increased with soaking times longer than 2 h. Thus, incubation for 2 h at 5 C resulted in optimium recovery of xanthomonads from rice seed. The pH of the seed solution remained at 7.0–7.2 after extraction and soaking.

Recovery of *X. o. oryzae* and *oryzicola* from artificially infested seed extracts. Recovery of target bacteria added to extracts from pathogen-free rice seed was compared on XOS and MXO. The Chinese strains, in particular, grew poorly on MXO. Recovery on XOS compared to MXO was 228% for *X. o. oryzicola* strain

TABLE 2. Percentage of recovery of *Xanthomonas oryzae* pv. *oryzae*, *X. o. oryzicola*, and *Xanthomonas*-like strains on XOS basal medium in the presence of selective inhibitors and on XOS and MXO[x]

Media[y]	*X. o. oryzae*			*X. o. oryzicola*		*Xanthomonas*-like spp.		
	HLT-69	ZHE-173	NX42	S1358	S44	X1-1	NP1	NP2
XOS basal +								
Cycloheximide	88	82	103	...[z]	...	...	...	...
Cephalexin	90	74	106	...	...	...	...	...
Kasugamycin	92	63	87	...	...	...	...	...
Methyl violet 2B	102	77	97	...	...	...	...	...
MXO	27	23	17	19	21	15	25	22
XOS	84	90	76	89	92	94	81	83

[x]Recovery = colony-forming units on test medium per colony-forming units on XOS basal medium × 100. Based on 6–10 plates per strain.
[y]Concentrations of cycloheximide, cephalexin, kasugamycin, and methyl violet were 100 mg/ml, 20 mg/ml, and 0.3 μg/ml, respectively.
[z]Not determined.

44, and 300 and 350% for the *X. o. oryzae* strains ZHE-173 and HLT-69, respectively. Colony diameters of target bacteria at similar population levels were similar on both XOS and MXO, averaging about 2.2 mm after 4 days at 28 C. However, MXO was more suppressive than XOS to the growth of saprophytic bacteria from seed. Mean recovery of saprophytic bacteria on XOS was 110% greater than on MXO. The increase in saprophytic bacteria counts on XOS was normally due to the failure to inhibit *E. herbicola* and a *Pseudomonas* spp. Regardless of the greater number of saprophytic bacteria on XOS, colony diameters remained small enough to allow easy detection of the target species (Fig. 1). Incubation times longer than 4 days did not result in increased populations of either saprophytic bacteria or target xanthomonads. In comparison, no *Xanthomonas* colonies were detected from dilution plating of infested seed extracts onto XOS basal medium due to significant interference by saprophytic microorganisms.

Recovery of xanthomonads from rice seed collected from infected plants. Recovery of *Xanthomonas* and xanthomonad-like bacteria was significantly greater on XOS than on MXO at all levels of seed infestation (Table 3). However, MXO was more effective in reducing the growth of saprophytic bacteria associated with rice seed (Table 3). XOS basal medium was totally unsatisfactory for the recovery of xanthomonads from rice seed extracts due to overgrowth of nontarget microorganisms.

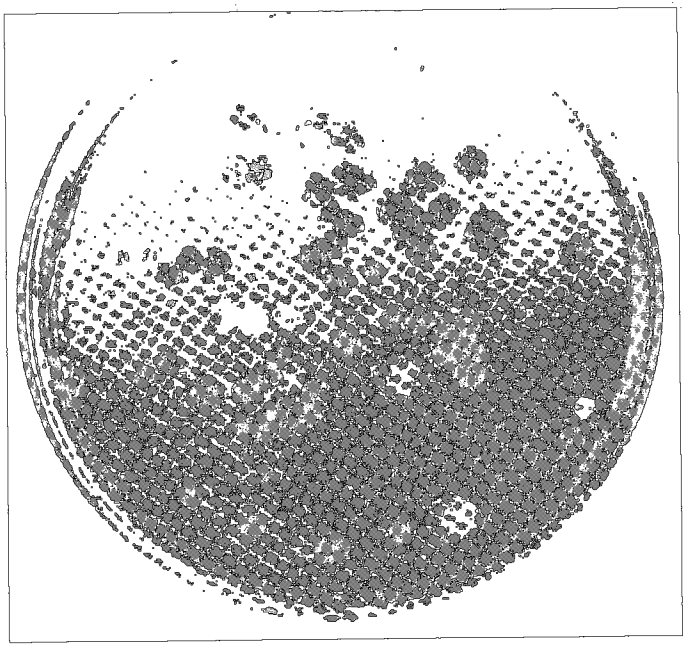

Fig. 1. Growth of *Xanthomonas oryzae* pv. *oryzae* (strain ZHE-173) from rice seed extract on XOS. Cells of *X. o. oryzae* were added to the rice seed extract. Arrow points to a typical *X. o. oryzae* colony after 4 days incubation at 28 C.

During the course of these recovery studies, pathogenicity studies were done on random colonies of Gram negative bacteria that showed typical *Xanthomonas* colony morphology and color. Of 30 *Xanthomonas*-like strains picked randomly from four Chinese rice seed lots and assayed for pathogenicity, all were nonpathogenic. At the same time positive symptoms developed on seedlings inoculated with known *X. o. oryzae* strains (Fig. 2). Bacteria similar to nonpathogenic *Xanthomonas* spp., strains X1-1 and X1-9 from Texas, also were isolated from California rice seed obtained from noninfected plants. Seed of one California lot contained 3.8×10^{-4} cfu/g of nonpathogenic *Xanthomonas*-like bacteria.

Inoculation with the *Xanthomonas*-like bacteria from Chinese seed resulted in pinpoint lesions of less than 1 mm diameter at the point of inoculation, and the spots never became water soaked. In comparison, pathogenic strains induced water-soaked lesions that later became yellow and brown, and the leaves were severely curled. Furthermore, the plants normally wilted. These characteristic lesions spread from the point of inoculation toward the leaf tip and base. Bacterial streaming was readily observable from the cut ends of infected leaves. Symptoms on plants inoculated with the low virulence Texas strain (X1-8) were similar to those induced by Chinese virulent strains except that lesion size was significantly reduced. Lesions ranged from 3 to 8.5 cm (mean 6.5 cm) with the Texas strain in contrast to 10–16 cm (mean 14 cm) with the virulent Chinese strains 14 days post-inoculation.

Nonpathogenic xanthomonad-like strains from California and Chinese rice seed, the low virulence strain (X1-8), and nonpathogenic strains from Texas were indistinguishable with respect to colony morphology and color from highly virulent Asian strains on YDC, NBY, PSA, XOS, and XOS basal media. Also, recovery of nonpathogenic *Xanthomonas*-like strains was comparable to the low virulence strain (X1-8) and *X. o. oryzae* strains from Asia on the various media that were evaluated by dilution plating assays using pure cultures (Tables 1, 2, and 4). The RNA from all suspected *Xanthomonas* strains, including nonpathogenic ones, hybridized comparably with a DNA probe based on *Xanthomonas*-specific 16s rRNA sequences (Fig. 3). RNA from all known non-xanthomonad species tested did not hybridize with the probe. In addition, spectrophotometric analyses of crude pigment extracts from the nonpathogenic and pathogenic xanthomonad strains showed the same absorption maxima of 443 nm. Thin-layer chromatography of pigments indicated R_f (retardation factor) values of 0.45–0.48 for major pigment spots, which is indicative of xanthomonadin. In contrast, analysis of pigments from a *E. herbicola* strain from rice seed extracts showed that xanthomonadin was not present.

DISCUSSION

Xanthomonas-like bacteria were detected from Chinese and California rice seed using a procedure that combines extraction and semi-selective isolation phases. The procedure was optimized for the detection of low populations of slow growing *X. o. oryzae* in the presence of faster growing microorganisms that are present

TABLE 3. Recovery of *Xanthomonas*-like bacteria from rice seed mixes

Medium	Number of seeds[x]		Colony-forming units recovered per gram of seed[y]	
	Healthy	Contaminated	*Xanthomonas*-like spp.	Saprophytes
MXO	495	5	0[z]	8.5×10^3 a
XOS			2.0×10^2 a	1.9×10^5 c
MXO	450	50	1.6×10^2 a	4.6×10^4 b
XOS			7.8×10^3 b	2.5×10^5 c
MXO	250	250	5.0×10^3 b	5.5×10^4 b
XOS			2.4×10^4 c	3.2×10^5 c

[x] Varying levels of seed infestation were obtained by mixing seed obtained from plants with severe symptoms of leaf blight (infested) with seed free of *Xanthomonas*-like bacteria.

[y] Recovery based on mean of five plates per seed assay.

[z] Means followed by the same letter in each column are not significantly different from one another at 0.5% level using Duncan's multiple range test.

at high populations in seed. However, in the seed lots tested, no pathogenic *X. o. oryzae* strains were detected.

Extraction of target bacteria from rice seed with minimum influence from contaminating microorganisms and seed constituents was based on optimizing the time of extraction, temperature, and time of soaking phases in initial extraction procedures. Our results agree with those of Fatmi and Schaad (7) in that the Stomacher technique effectively opens the seed without complete maceration. In contrast to *C. m. michiganensis* (7), a brief 2-h incubation at 5 C resulted in an increased number of target cells. Longer extraction times increase the interference from seed constituents, and longer soaking times or incubation at 25 C actually reduced recovery of the target bacteria. Passive soaking of seed resulted in decreased recovery of xanthomonads compared to extraction before a soaking phase.

XOS basal medium allowed optimium recovery of xanthomonads in dilution plating assays using pure cultures. However, significant interference resulted from nontarget microorganisms in rice seed extracts. Added chemical inhibitors decreased the majority of contaminating bacterial flora from seed extracts. Although *E. herbicola* and a *Pseudomonas* spp. were not appreciably inhibited on XOS, the selective inhibitors suppressed growth rates of these bacteria enough so that the slower growing target bacteria could be observed. The selective inhibitors affected the recovery of *X. o. oryzae* and *X. o. oryzicola* strains. Thus, a compromise in the inhibitory chemical concentrations was necessary to assure maximium recovery of all strain types. In particular, cephalexin was very effective in inhibiting non-*Xanthomonas* strains from seed. Most Chinese strains of *X. o. oryzae* we tested failed to grow well on MXO, a selective medium developed for the Texas strains of *X. o. oryzae* (11). MXO contains methyl green and gentamycin, which we found to severely inhibit known strains of *X. o. oryzae* and *X. o. oryzicola* from Asia. Such results suggest that the Texas and Asian strains may be different bacteria. In comparative assays, recovery of rice xanthomonads from seed was significantly greater on XOS than on MXO medium. Preliminary tests in China also show that XOS is superior to MXO for the recovery of *Xanthomonas* spp. from infected rice leaves (H. Ye, *unpublished data*). MXO did perform somewhat better for the isolation of target bacteria from diseased tissues than for isolation from seed extracts. This is most likely due to the very high populations of the pathogen in leaf lesions. When numbers of target bacteria are low in comparison to other bacteria, such as in seed, XOS is clearly superior. Further improvements of XOS could be made by adding compounds inhibitory to *Pseudomonas* spp. and *E. herbicola*.

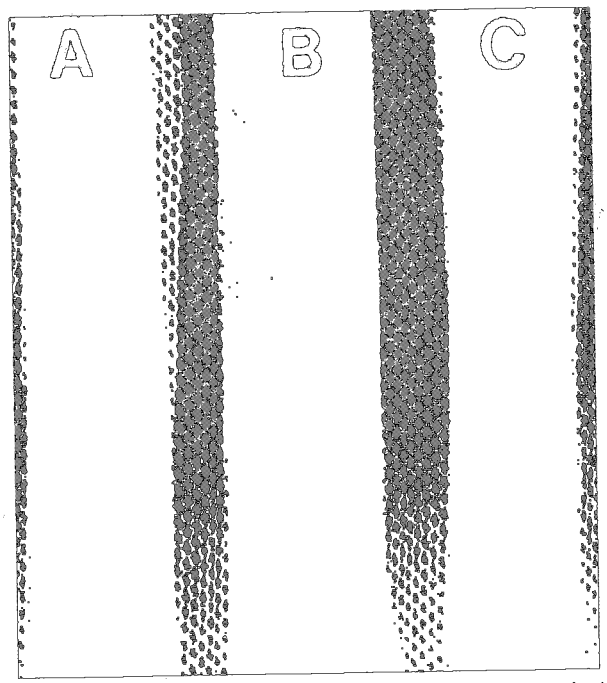

Fig. 2. Rice leaves with typical symptoms (14 days post-inoculation) of bacterial leaf blight following needle inoculation with a nonpathogenic *Xanthomonas*-like strain (NP1) A, from rice seed from China; B, a Chinese strain (HN-31) of *Xanthomonas oryzae* pv. *oryzae*; and C, a low virulence *X. o. oryzae* strain (X1-8) from Texas.

Nonpathogenic *Xanthomonas*-like bacteria were common on XOS from rice seed obtained from China and California. In a limited survey of seed lots, including ones collected from plants grown in China and severely infected with *X. o. oryzae*, xanthomonad-like colonies were isolated but none were pathogenic when inoculated on a susceptible rice cultivar. Our failure to detect *X. o. oryzae* from seed extracts may be due to several reasons including: limited number of seed lots assayed, high populations of nonpathogenic xanthomonads present, or rapid death of *X. o. oryzae* in seed. The latter is suggested by the ease in isolation of the pathogen from seed harvested from symptomatic plants within 15–30 days after harvest (H. Ye, *unpublished data*). According to Y. Shen, associate professor of Plant Protection (China National Rice Research Institute, Hangzhou, China), the seed we assayed was collected from plants with severe symptoms of leaf blight. However, our assays were done more than 9 mo after harvest. The pathogen could have died or lost virulence in that time. The nonpathogenic strains were visually indistinguishable in terms of colony morphology, growth, and

TABLE 4. Percentage of recovery of *Xanthomonas oryzae* pv. *oryzae*, *X. o. oryzicola*, and *Xanthomonas*-like bacteria on different growth media

Strain	NGA[x]	SUWA	NBY	PSA	WFP	XOS basal	YDC
X. o. oryzae							
HX42	100[y]	105	105	105	109	120	87
HN31	100	116	93	97	120	119	73
LN844	100	118	104	100	104	114	58
JS262	100	130	109	126	123	146	107
ZHE173	100	117	110	100	118	121	65
X1-8	100	...[z]	...	111	...	115	...
X. o. oryzicola							
S44	100	...	...	100	...	112	...
S1358	100	...	...	100	...	121	50
Nonpathogenic *Xanthomonas*-like spp.							
X1-1	100	...	...	84	...	113	65
NP1	100	...	...	110	...	112	78
NP2	100	...	...	100	...	138	...
X1-9	100	...	...	112	...	123	...

[x]Abbreviations: NGA, nutrient glucose agar (23); SUWA, (24); NBY, nutrient broth yeast extract agar (26); PSA, peptone sucrose agar (12); WFP, Wakimoto's medium (18); XOS basal medium; YDC, yeast extract dextrose calcium carbonate agar (23).

[y]Cultures grown for 18 h in nutrient sucrose broth were diluted to 10^{-5} and 10^{-6} sterile saline, plated on the media, and incubated for 4 days at 28 C. Mean recovery = on the test medium per colony-forming units on NGA × 100.

[z]Not determined.

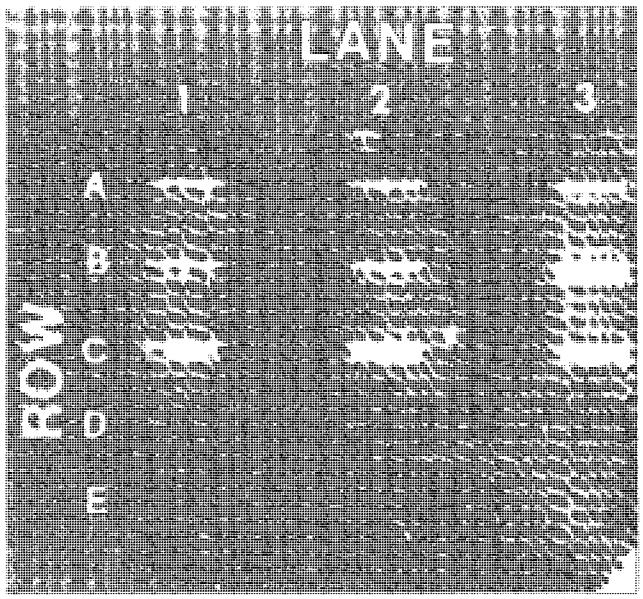

Fig. 3. Autoradiogram of nucleic acid hybridizations. RNA was isolated from bacteria according to DeParasis and Roth (5) and 2 μg was blotted onto a Hybond N filter. The filter was probed with a [^{32}P]-end labeled oligonucleotide complementary to *Xanthomonas*-specific 16s rRNA sequences (5). Prehybridization, hybridization, and washing were done according to Bragg and Bollon (3). A, nonpathogenic *Xanthomonas*-like bacteria X1-1, NP-1, NP-2; B, *X. oryzae* pv. *oryzae* X1-8, HN-31, LN-844; C, *X. o. oryzicola* S44 and S1358 and *X. o. oryzae* PXO61; D, *Erwinia herbicola* RS1, *E. herbicola* RS2, *Pseudomonas* spp. from rice seed; E, *E. chrysanthemi*, *P. syringae* pv. *phaseolicola*, *P. s. tomato*.

color from known Chinese strains of *X. o. oryzae*. Analyses of crude pigment extracts from these nonpathogenic strains suggested that xanthomonadin was present. Further, the strains hybridized to a *Xanthomonas*-specific DNA probe. Thus, the nonpathogenic strains from rice seed belong to the genus *Xanthomonas*, but whether they are closely related to *X. o. oryzae* will need additional testing. Nonpathogenic *Xanthomonas* spp. have been previously isolated from rice in Texas and Louisiana (2,14). Serological assays indicate they are more related to *X. o. oryzicola* than to *X. o. oryzae* types (2; H. Ye, *unpublished data*). The presence of nonpathogenic, atypical xanthomonads in rice seed lots could present a potential identification problem for regulatory agencies. In the past, seedborne, nonpathogenic xanthomonads may have been confused with *X. o. oryzae* or *X. o. oryzicola*. Similar situations exist with citrus nursery stocks in Florida (8,9) and tomato transplants in Georgia (10). Gitaitis et al (10) isolated nonpathogenic xanthomonads from tomato and pepper seedlings and suggested that, in some cases, transplants may have been denied certification due to the presence of these strains. Rejection of seed or plants should be based on the presence of a regulated pathogen not a closely related nonpathogen (22).

The recovery of yellow-pigmented *Xanthomonas*-like bacteria from rice seed does not necessarily suggest the presence of *X. o. oryzae* or *X. o. oryzicola*. These results illustrate the importance of doing pathogenicity assays to confirm the identity of *Xanthomonas* strains, in particular, considering the economic importance of rice and existing regulatory constraints. Further experiments are underway to determine the relationship of the *Xanthomonas*-like strains and *X. o. oryzae* from Asia.

LITERATURE CITED

1. Aldrick, S. J., Buddenhagen, I. W., and Reddy, A. P. K. 1973. The occurrence of bacterial leaf blight in wild and cultivated rice in northern Australia. Austral. J. Agric. Res. 24:219-227.
2. Benedict, A. A., Alvarez, A. M., Berestecky, J., Imanaka, W., Mizumoto, C. Y., Pollard, L. W., Mew, T. W., and Gonzalez, C. F. 1989. Pathovar-specific monoclonal antibodies for *Xanthomonas campestris* pv. *oryzae* and for *Xanthomonas campestris* pv. *oryzicola*. Phytopathology 79:322-328.
3. Bragg, P. W., and Bollon, A. P. 1985. Comparison of oligonucleotide and long DNA fragments as probes in DNA and RNA dot, Southern, northern, colony and plaque hybridizations. BioTechniques 3:208-220.
4. California Department of Food and Agriculture. 1983. Federal Quarantine Number 55. California Plant Quarantine Manual.
5. DeParasis, J., and Roth, D. A. 1990. Nucleic acid probes for identification of phytobacteria: Identification of genus-specific 16s rRNA sequences. Phytopathology 80:618-621.
6. Eamchit, S., and Mew, T. W. 1982. Comparison of virulence of *Xanthomonas campestris* pv. *oryzae* in Thailand and the Philippines. Plant Dis. 66:556-559.
7. Fatmi, M., and Schaad, M. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology 78:121-126.
8. Gabriel, D. W., Kingsley, M. T., Hunter, J. E., and Gottwald, T. 1989. Reinstatement of *Xanthomonas citri* (ex Hasse) and *X. phaseoli* (ex Smith) to species and reclassification of all *X. campestris* pv. *citri* strains. Int. J. Sys. Bacteriol. 39:12-22.
9. Graham, J. H., Hartung, J. S., Stall, R. E., and Chase, A. R. 1990. Pathological, restriction-fragment length polymorphism, and fatty acid profile relationships between *Xanthomonas campestris* from citrus and noncitrus hosts. Phytopathology 80:829-836.
10. Gitaitis, R. D., Sasser, M. J., Beaver, R. W., McInnes, T. B., and Stall, R. E. 1987. Pectolytic xanthomonads in mixed infections with *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper transplants. Phytopathology 77:611-615.
11. Gonzalez, C. F., Xu, G. -W., Li, H. -L., and Cosper, J. W. 1991. *Leersia hexandra*, an alternate host for *Xanthomonas campestris* pv. *oryzae* in Texas. Plant Dis. 75:159-162.
12. Hayward, A. C. 1960. A method for characterizing *Pseudomonas solanacearum*. Nature (London) 186:405-406.
13. Irey, M. S., and Stall, R. E. 1981. Value of xanthomonadins for identification of pigmented *Xanthomonas campestris* pathovars. Pages 85-95 in: Proc. Int. Conf. Plant Pathog. Bact. 5th.
14. Jones, R. K., Barnes, L. W., Gonzalez, C. F., Leach, J. E., Alvarez, A. M., and Benedict, A. A. 1989. Identification of low virulence strains of *Xanthomonas campestris* pv. *oryzae* from rice in the United States. Phytopathology 79:984-990.
15. Lozano, J. C. 1977. Identification of bacterial leaf blight in rice caused by *Xanthomonas oryzae* in America. Plant Dis. Rep. 61:644-648.
16. Mew, T. W. 1987. Current status and future prospects of research on bacterial blight of rice. Annu. Rev. Phytopathol. 25:359-382.
17. Mizukami, T., and Wakimoto, S. 1969. Epidemiology and control of bacterial leaf blight of rice. Annu. Rev. Phytopathol. 7:51-72.
18. Ou, S. H. 1985. Rice Diseases. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England. 380 pp.
19. Randhawa, P. S., and Schaad, N. W. 1984. Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. Phytopathology 74:268-272.
20. Reckhaus, P. M. 1983. Occurrence of bacterial blight of rice in Niger, West Africa. Plant Dis. 67:1039.
21. Roth, D. A. 1989. Review of extraction and isolation methods. Pages 3-8 in: Detection of Bacteria in Seed and Other Planting Material. A. W. Saettler, N. W. Schaad, and D. A. Roth, eds. The American Phytopathological Society, St. Paul, MN. 127 pp.
22. Saettler, A. W. 1989. The need for detection assays. Pages 1-2 in: Detection of Bacteria in Seed and Other Planting Material. A. W. Saettler, N. W. Schaad, and D. A. Roth, eds. The American Phytopathological Society, St. Paul, MN. 127 pp.
23. Schaad, N. W. 1988. A Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. The American Phytopathological Society, St. Paul, MN. 164 pp.
24. Suwa, T. 1962. Studies on the culture media of *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson the causal organism of bacterial blight of rice plant. Ann. Phytopathol. Soc. Jpn 27:165-171.
25. Swings, J., Van den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T. W., and Kersters, K. 1990. Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris* pv. *oryzae*) and bacterial leaf streak (*Xanthomonas campestris* pv. *oryzicola*) of rice as pathovars of *Xanthomonas oryzae* (ex Ishiyama 1922) sp. nov., nom. rev. Int. J. Syst. Bacteriol. 40:309-311.
26. Vidaver, A. K. 1989. Gram positive bacteria. Pages 12-16 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. N. W. Schaad, ed. The American Phytopathological Society, St. Paul, MN. 164 pp.
27. Washington, J. A. 1974. Laboratory procedures in clinical microbiology. Little, Brown and Co., Ltd. Boston, MA. 522 pp.